Confirmation of the elimination of *Apple stem grooving virus* from apple trees by *in vitro* chemotherapy

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Abstract

Apple stem grooving virus (ASGV) is widespread in its distribution in apple trees. The virus causes tree decline and graft union necrosis in certain combinations of scion and rootstock, and attempts are made usually to control the virus where apples are grown commercially. ASGV is difficult if not impossible to eliminate by heat therapy. In 1996, *in vitro* cultures of apple infected with ASGV were grown for 9-12 weeks on media containing quercetin and ribavirin (10 µg/mL of each), then cultured on media free of these chemicals. Analysis by immunocapture (IC) RT-PCR failed to detect the presence of ASGV, while all untreated controls were positive. Treated and untreated cultures were subcloned, rooted, hardened, and eventually planted in the field. The plants were observed and tested annually by IC/RT-PCR from 1998 - 2008. The treated plants were consistently negative by IC/RT-PCR, while untreated plants tested positive for ASGV. After 11 years of testing by the sensitive IC/RT-PCR assay it is safe to say that *in vitro* chemotherapy with quercetin and ribavirin is effective for the elimination of ASGV from apple.

Keywords: *Apple stem grooving virus*, *in vitro* chemotherapy, quercetin, ribavirin, immunocapture RT-PCR

Introduction

Apple stem grooving virus (ASGV) is an important target in any *Malus* (apple) virus certification program. The virus is widely distributed and is transmitted by budding or grafting, so use of healthy propagating material is essential for controlling the movement of the virus (Nemeth, 1986; Welsh and van der Meer, 1989). ASGV is symptomless in most commercial cultivars (Welsh and van der Meer, 1989), but may cause tree decline and graft union necrosis in susceptible scion/rootstock combinations (Yanase et al. 1990). Since ASGV infection is common, certain apple cultivars may not be readily available free of ASGV infection. In this case virus therapy may be considered. ASGV has been described as one of the most difficult viruses to eliminate by any procedure (Knapp et al., 1995a, b). Reports of ASGV elimination from apple and citrus by heat therapy (Campbell, 1968; Miyakawa, 1980) have not been confirmed by long term confirmatory testing using sensitive diagnostic assays. Heat therapy is one of several methods used for plant virus elimination which includes; shoot tip culture, meristem tip culture, chemotherapy, thermotherapy, or various combinations of the above (Spiegel et al., 1993).

James et al. (1997) claimed successful elimination of ASGV by using a combination of the antiviral chemicals quercetin and ribavirin. Shoot tip cultures of ASGV-infected apple and shoot tip cultures of ASGV-infected Nicotiana occidentalis (a herbaceous host) were treated by in vitro chemotherapy using ribavirin and quercetin at a concentration of 10 µg/mL of each. The plants were exposed to the chemicals for a period of 9-12 weeks, and subsequent testing by ISEM, herbaceous host indexing, RT-PCR, and immunocapture RT-PCR did not detect ASGV in any culture subjected to this treatment (James et al., 1997). Untreated control plants were all positive. Attempts at plant virus elimination may suppress the concentration of a virus to levels that are not detectable. Positive results may be obtained if the plants are grown for an extended period of time subsequent to being treated, then re-tested (Knapp et al. 1995b). To confirm virus elimination it is essential therefore that treated plants be propagated for an extended period, free of any antiviral chemicals in the case of chemotherapy, and delayed testing carried out to allow replication and detection of any virus particles that may have remained after treatment (Hansen, 1989). This is especially important in woody fruit trees where: a) virus replication and recovery may be slow, and b) virus detection is inefficient due to the presence of inhibitors. The use of sensitive diagnostic assays improves the validity of claims of virus elimination (James et al. 1997).

In this study apple cultures that gave negative results by immunocpature RT-PCR after *in vitro* chemotherapy were subcloned, rooted, hardened, and eventually planted in the field. The results of annual IC/RT-PCR testing of the treated plants which were maintained in the field for over 10 years confirm the elimination of ASGV.

Materials and methods

Field propagation: Shoot cultures that were treated for 9 – 12 weeks with quercetin and ribavirin (10 µg/mL of each), and gave negative results when tested by IC/RT-PCR, were rooted and planted in the field in 1997 (James, 2001). Untreated cultures that gave positive results were rooted and planted also, as positive controls. There were a total of 15 trees (9 derived from treated cultures, and 5 derived from untreated cultures) and these were planted randomly with no indication of plant status. They were identified by a number only (#1 - #15, Table 1).

Tab. 1 IC/RT-PCR analysis from 1998 – 2008 (11 years) of apple trees derived from *in vitro* cultures treated with quercetin and ribavirin (10 µg/mL of each), and untreated positive controls.

Tree #[A]	***Status***[B]	IC/RT-PCR Resultc[C]
1	T	-
2	T	-
3	UT	+
4	T	-
5	T	-
6	UT	+
7	UT	+
8	T	-
9	UT	+
10	UT	+
11	T	-
12	T	-
13	UT	+
14	T	-
15	T	-

[A] Trees derived from treated and untreated *in vitro* cultures were planted randomly and identified by numbers only for purposes of testing; [B] T = trees derived from *in vitro* cultures treated with quercetin and ribavirin (10 µg/mL of each), UT = Trees derived from *in vitro* cultures that were untreated positive controls; [C] Consistent results were obtained for the annual testing conducted from 1998 – 2008; - = negative, and + = positive.

The plants were included in the regular schedule of maintenance of field grown apple trees maintained at the Sidney Laboratory - Centre for Plant Health. The plants were pruned annually and the row of trees top-dressed with a granular fertilizer. Pest control treatments were applied on a regular basis, including treatments for apple scab, apple codling moth, and powdery mildew. The plants were blind tested annually (May – June) by various technicians for the period 1998 – 2008. Healthy apple plants were used as negative controls. Also apple plants known to be infected with ASGV were included as additional positive controls.

Immunocapture (IC)RT-PCR: IC/RT-PCR was carried out using essentially procedure A as described by James (1999). ASGV polyclonal antiserum, cross-absorbed with clarified healthy *Chenopodium quinoa* sap (*C. quinoa* was the propagation host for the purified virus used for PAb production), was purified and adjusted to 2 µg/mL. This was diluted 1:100 and 100 µL added to each 0.5 ml microfuge tube. See James (1999) for further details.

Results

The results obtained by IC/RT-PCR testing for ASGV for the 11 year period from 1998 – 2008 were consistent. All 9 trees derived from *in vitro* cultures treated with a combination of ribavirin and quercetin (10 µg/mL each) gave negative results when tested for the presence of ASGV by IC/RT-PCR. The results obtained in 2008 are shown in Figure 1. The 5 trees derived from untreated *in vitro* cultures were consistently positive (Fig. 1, 2008 results). Additional controls were always included in each assay. These included known infected plants (Fig. 1A and B, indicated as +), healthy apple (Fig 1B), and water controls (Fig 1B). The results expected with these controls were observed.

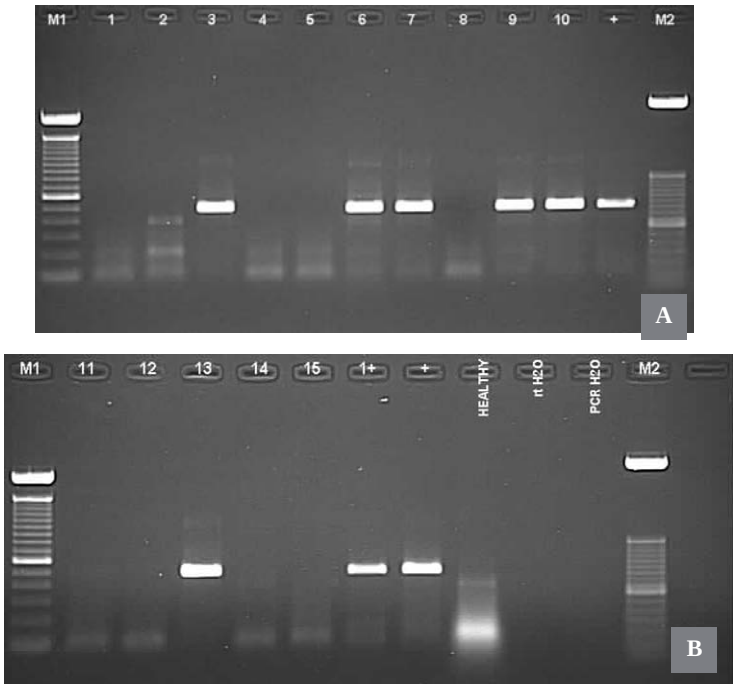

Fig. 1 Agarose gel analysis of the products of an IC/RT-PCR assay performed in 2008, of 15 randomly planted apple trees derived from *in vitro* cultures treated with quercetin and ribavirin, 10 µg/mL of each (Fig. 1A, lanes 1, 2, 4, 5, 8; Fig. 1B, lanes 11, 12, 14, 15); and apple trees derived from untreated positive controls (Fig. 1A, lanes 3, 6, 7, 9, 10; Fig. 1B lane 13). Additional positive controls (+) and water controls were included also.

Discussion

The wide distribution of ASGV (Nemeth, 1986; van der Meer, 1989) means that desirable virus-free germplasm may not always be available. Under such conditions having a reliable procedure for virus elimination may prevent the need to destroy valuable material and reduce the likely hood of planting infected material. ASGV is difficult to eliminate (Knapp et al., 1995a & b) and no well validate procedure for the elimination of ASGV has been described. ASGV elimination was claimed by James et al. (1997). Evidence of elimination was obtained for ASGV infected apple and *N. occidentalis*. In this study testing was conducted annually, over an 11 year period (1998 – 2008), by IC/RT-PCR. IC/RT-PCR is a very sensitive assay (Candresse et al., 1994; James 1999), and the use of random blind samples in this study provides increased confidence in the results. Eleven years of testing provided consistent results where the trees derived from cultures treated with quercetin and ribavirin remained negative, while trees derived from untreated cultures gave positive results consistently. The accumulated data confirms that virus elimination was achieved.

Ribavirin (1-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is a base anolog of adenine or guanine and was developed specifically for use as an antiviral chemical (Hansen, 1989). Several modes of action have been hypothesized including the disruption of virus replication; by inhibiting nucleic acid synthesis, inhibiting RNA-dependent RNA polymerase, or inhibiting 5'-capping of viral RNA. Quercetin (3,5,7,3',4'-pentahydroxyflavone) is a natural product flavonoid. Flavonoids have been shown to be effective antivirals against several plant viruses (French et al., 1991; French and Towers, 1992; Malhotra et al., 1996). Flavonoids enhance cAMP levels by inhibiting cAMP phosphodiesterase (Mucsi and Pragai, 1985) and this may affect virus replication. Ribavirin has been effective against a range of viruses, but in many cases the results have been disappointing (Hansen, 1989). James et al. (1997) found that some cultures treated with only ribavirin gave negative results by RT-PCR, but were positive by IC/RT-PCR. Also the concentration of the

virus increased to RT-PCR detectable levels when cultures were grown on media free of ribavirin. Quercetin was found to be more effective than ribavirin in tomato ringspot virus (TomRSV) inhibition studies (Malhotra et al., 1996).

Trees derived from *in vitro* cultures treated with ribavirin and quercetin appeared normal, with normal flowers and fruits. There was no evidence of unusual phenotypic changes or abnormalities, further confirming the observations of James (2001).

This study confirms that the antiviral chemical combination of ribavirin and quercetin (10 µg/mL of each) was effective for the elimination of ASGV from apple. ASGV was eliminated from infected *N. occidentalis* cultures by this treatment (James et al., 1997), and so it is not host specific. Treatment for 9 – 12 weeks was effective, which agrees with the findings of Malhotra et al. (1996) who found that 12 weeks was optimal for quercetin inhibition of TomRSV. It would be interesting to determine if this combination of chemicals in *in vitro* chemotherapy is effective for the elimination of other plant viruses.

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